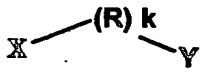
General Structure of Heterobifunctional Linkers

i 

where k = 1-100

R= Alkyl, cycloalkyl, cycloalkyl-alkyl, aromatic, alkyl-aromatic, stillbene, heterocyclic, alkyl-heterocyclic, CH_2CH_2-O-, alkyl-CH_2CH_2-O-alkyl, CH_2-CH=CH-, CH_2-NHCO, alkyl-NHCO-alkyl, CH_2CH_2-S-, CH_2CH_2-NH-, Long Chain Alkyl Amino, etc.

X = NH_2, succinimidyl, maleimidyl, iodoacetamido, bromoacetamido, thiol,

Y = Biotin

= Biotin/Avidin
= Biotin/Streptavidin (SA)
= Alkaline Phosphatase (AP)
= Casein
= beta-Lactamaze
= BSA
= IgG
=Avidin-AP
= Streptavidin-AP
= Biotin or Streptavidin complexed with :

Glycoproteins, enzymes, antibodies, DNA, RNA, peptides , derivatized particles made of polystyrene, nylon, gold, polyacrylamide, and other solid surfaces such as microtitre plates, glass (silicon) plates, and any other polymer comprised of active functions, for example, -OH, -NH2, -SH, succnimidym, maleimido groups.

Figure 1. General chemical structure and compositions of the heterobifunctional linkers of the Present Invention

Figure 2. Classification of Kinases and Phosphatases by Target Structure

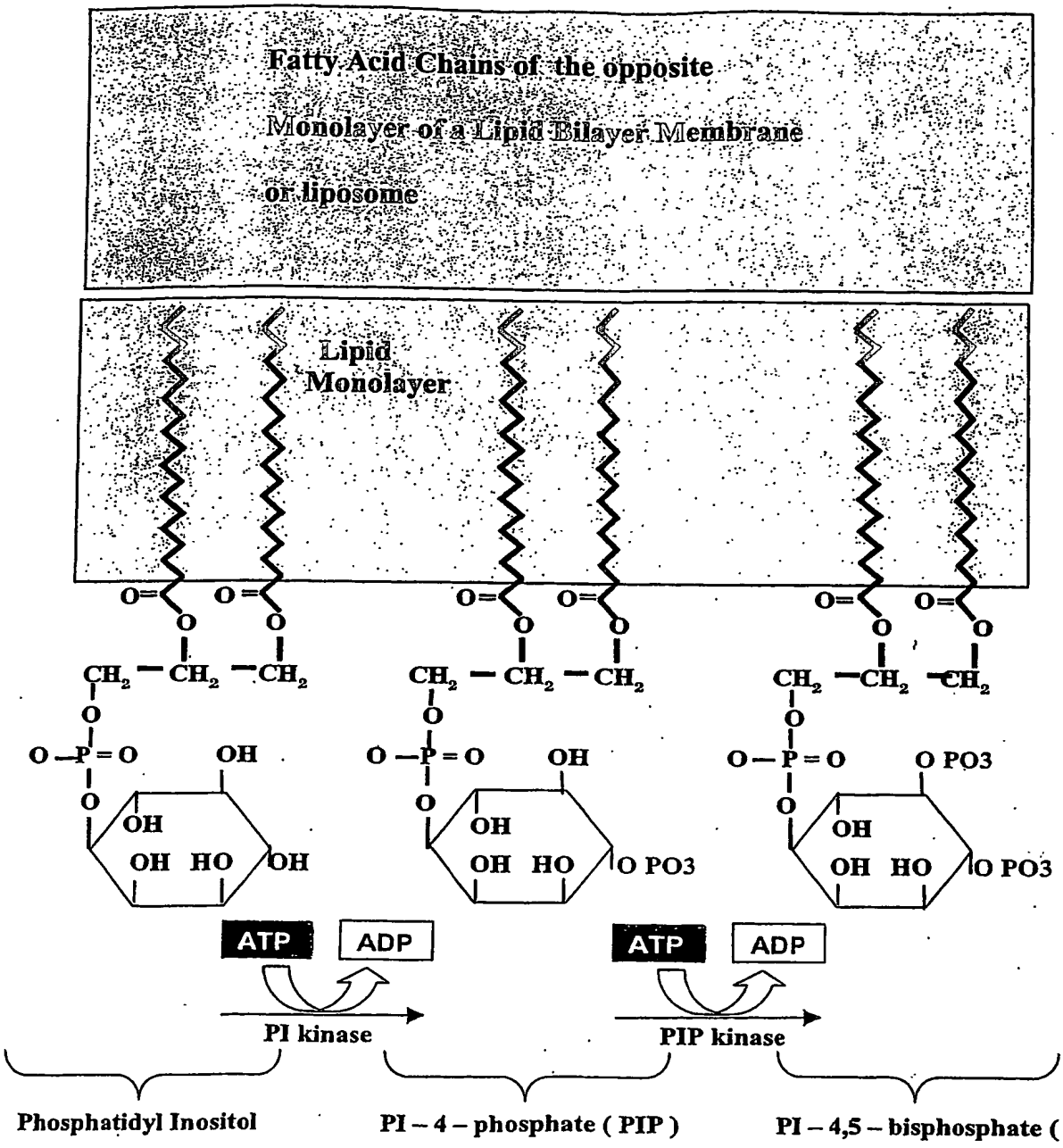
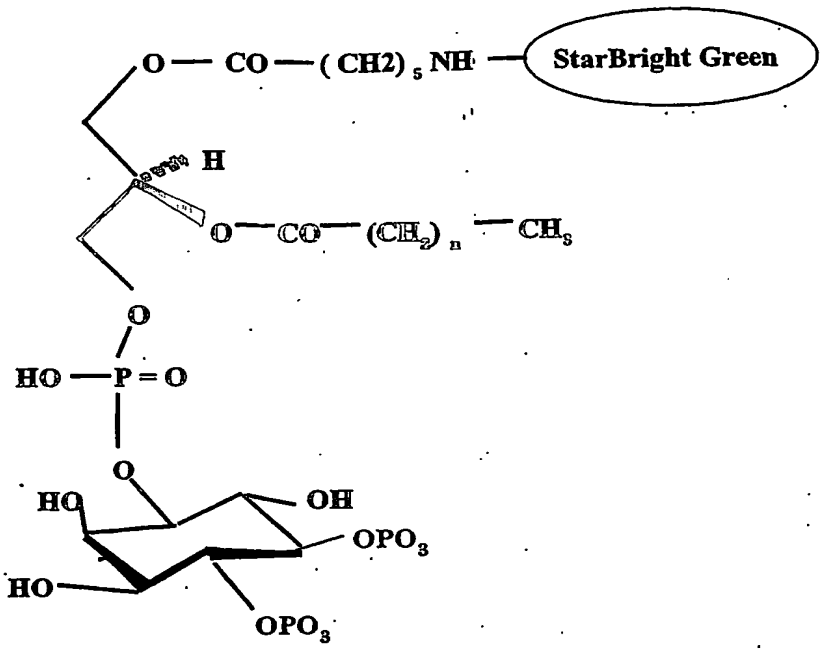


Figure 2a . a) representative water insoluble target and sites of specific actions of lipid kinases. Phosphatidyl Inositol and the Site specific actions of two lipid kinases



STARBRIGHT GREEN - PHOSPHATIDYLINOSITOL– 4,5- BISPHOSPHATE
[STARBRIGHT GREEN - PtdIns(4,5)P2]

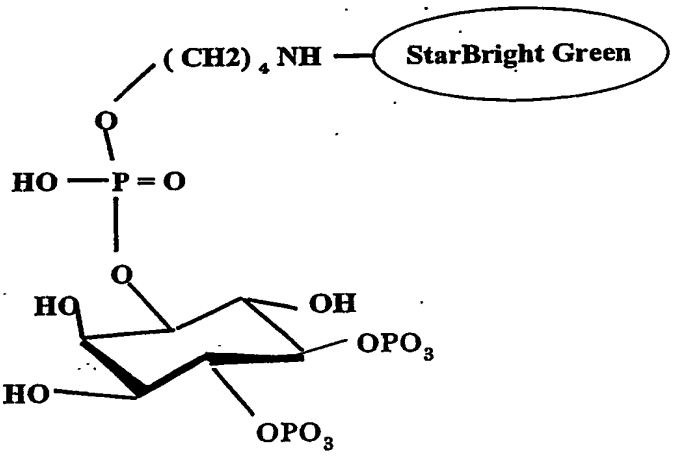


Figure 2.b. Water soluble lipid kinase target substrates: above, an example of the water soluble, StarBright-labeled derivatives of phosphatidyl inositol and its phosphorylated products. Alternative target substrates may be the single fatty acyl chain 1-StarBright Green –*myo* – inositol -1 phosphate lithium salts shown below and described in the text.

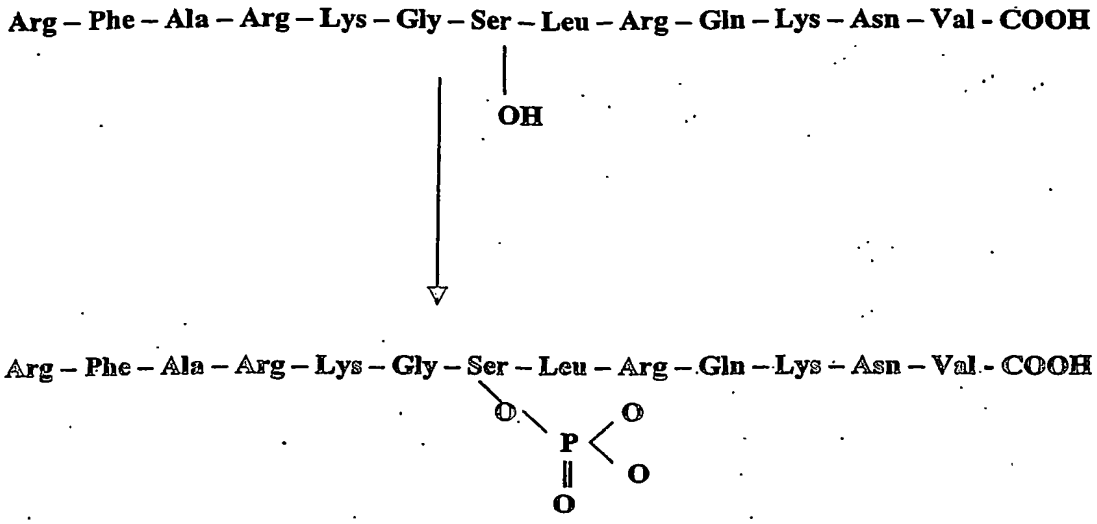


Figure 2. c. Peptide Target Substrate Phosphorylation -
The pseudosubstrate of Protein Kinase C-alpha and the site specific Phosphorylation of Serine by the PKC isozyme, PKC-theta

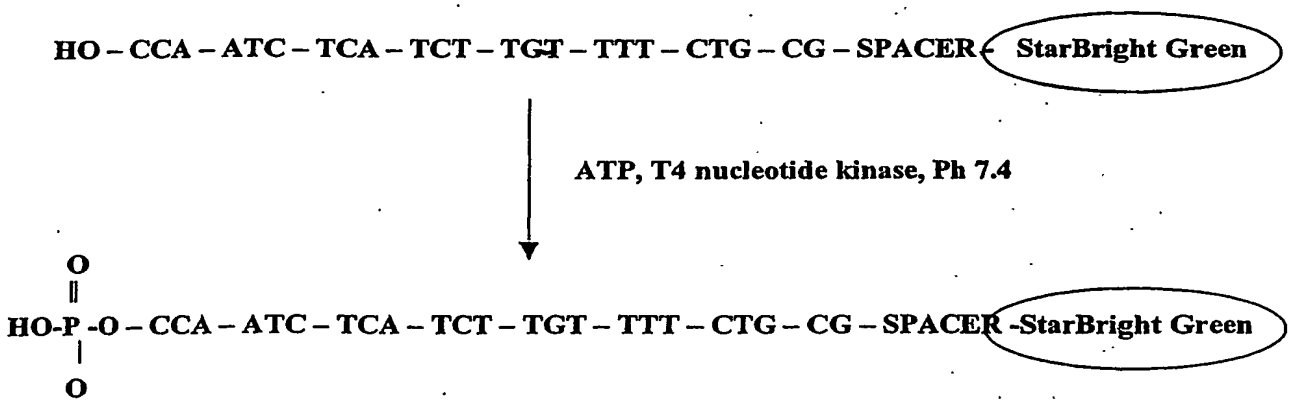
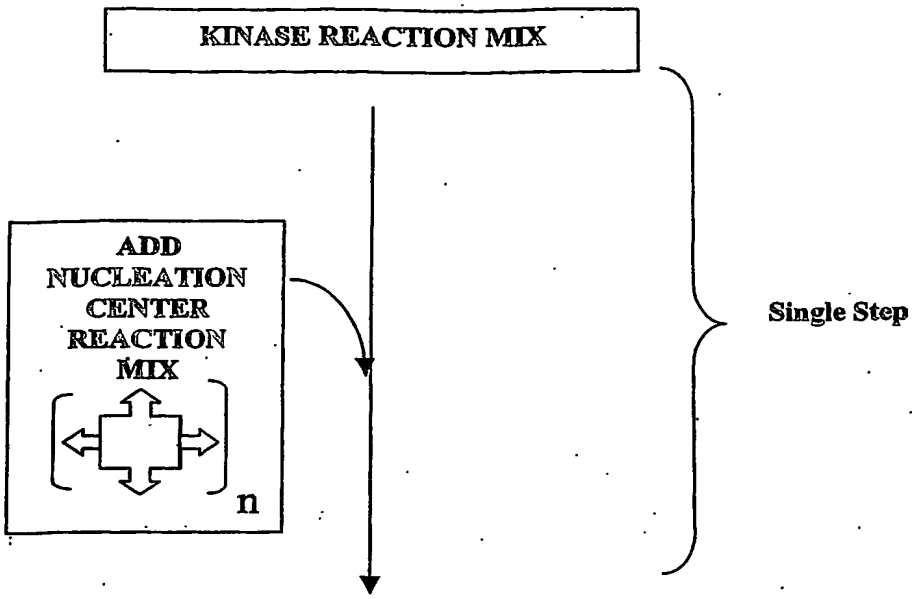
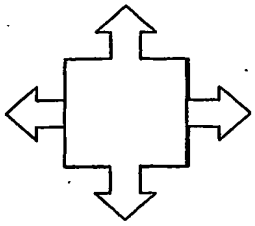


Figure 2.d. Oligonucleotide Target Substrate Phosphorylation –
The beta-actin target of T4 nucleotide kinase and the terminal phosphorylation of the oligonucleotide by the kinase

a) Single Step ***Homogeneous*** Assay using the rapid reaction method of the Present Invention



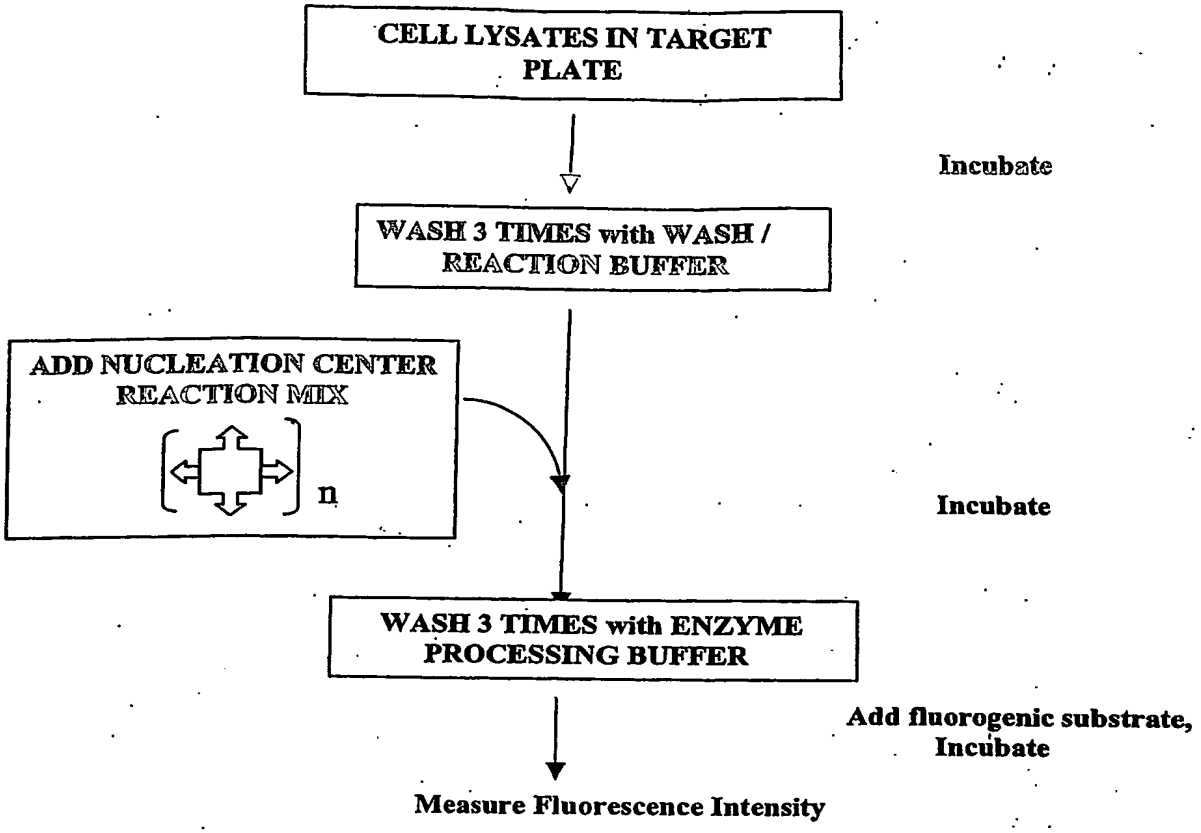
b) **the " Nucleation Effect "** in which multiple heterobifunctional linkers are attached to High Molecular weight core molecules such as avidin or another polymer to create a multi- valent reaction center that serves to enhance reaction rates,



where the square at the center represents the high molecular weight core that is conjugated to multiple copies (n > 2) of the heterobifunctional linkers (arrow heads) shown in Figure 1.

Figure 3. Schematic diagram (a) of the single step ***homogeneous*** assay method based upon the " nucleation effect " of the present invention and an idealized diagram (b) illustrating the nucleation effect itself;

a) Multi- Step *Heterogeneous* Assay of the Present Invention



b)

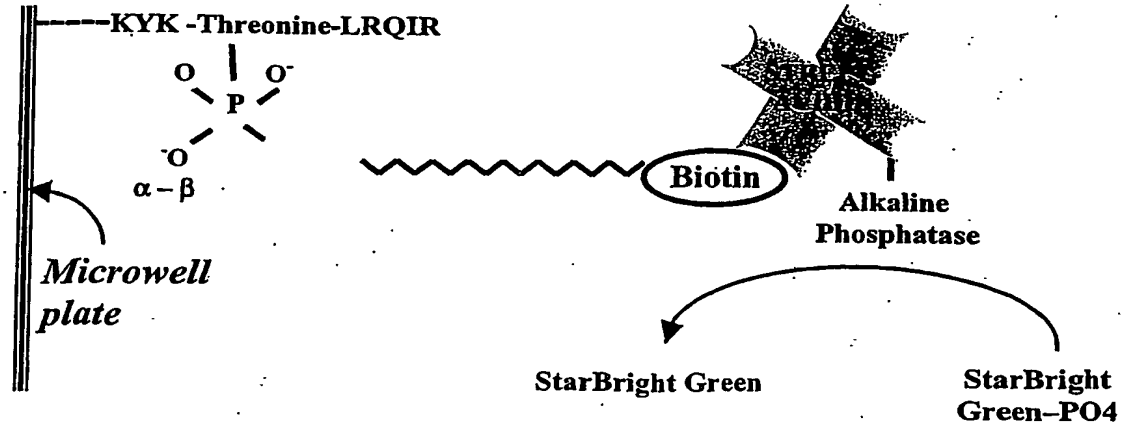


Figure 4. Schematic diagram (a) and mechanism (b) of the ***heterogeneous*** assay method based upon the nucleation effect of the present invention

Phosphoramidate Chemistry For Developing Fluorescence Polarization Based Protein Kinase Assays

Schematic Representation of Steps Involved:

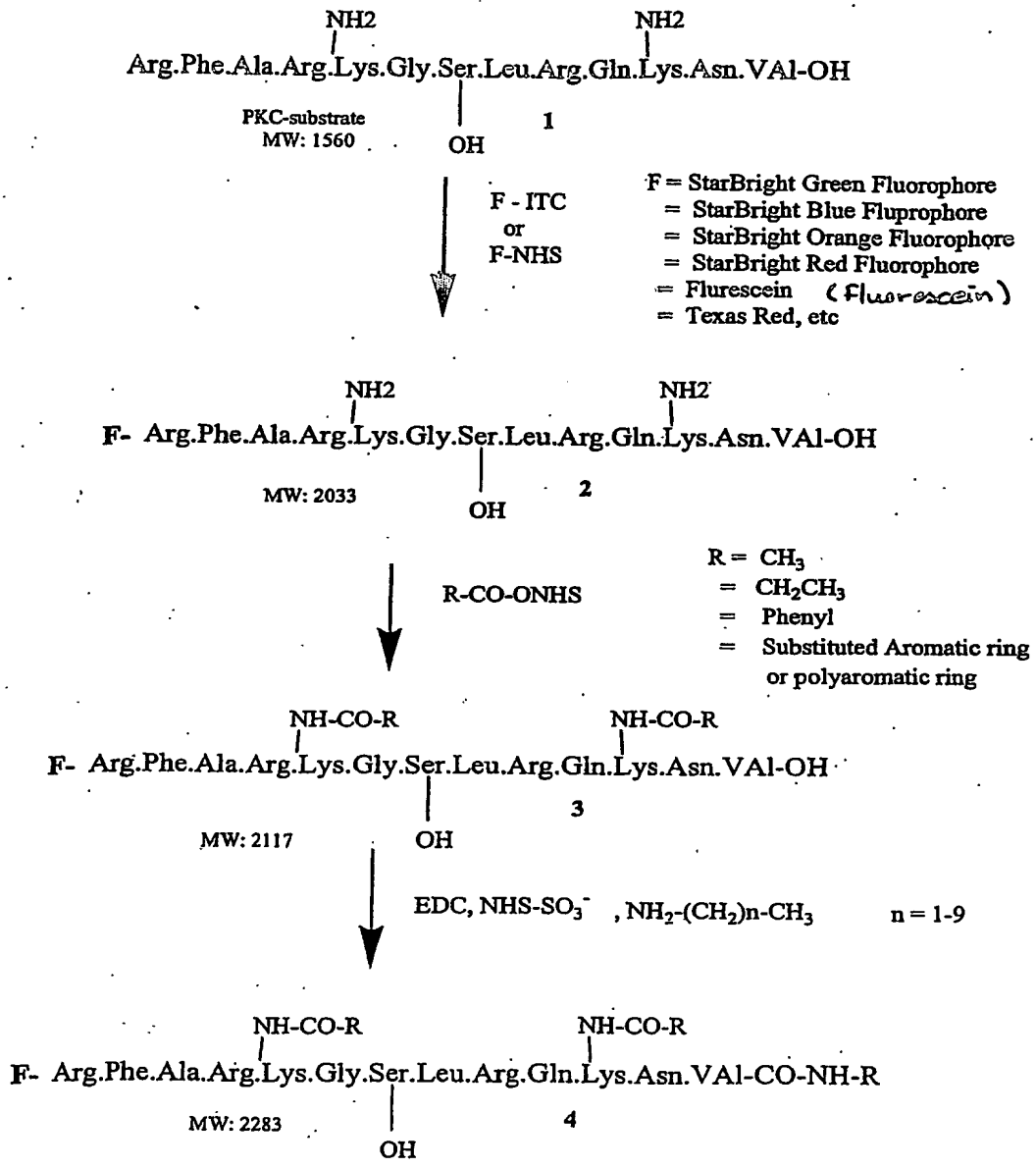


Figure 5. Novel protocols for blocking potentially reactive $-NH_2$ and $-COOH$ groups on peptide targets of the present invention

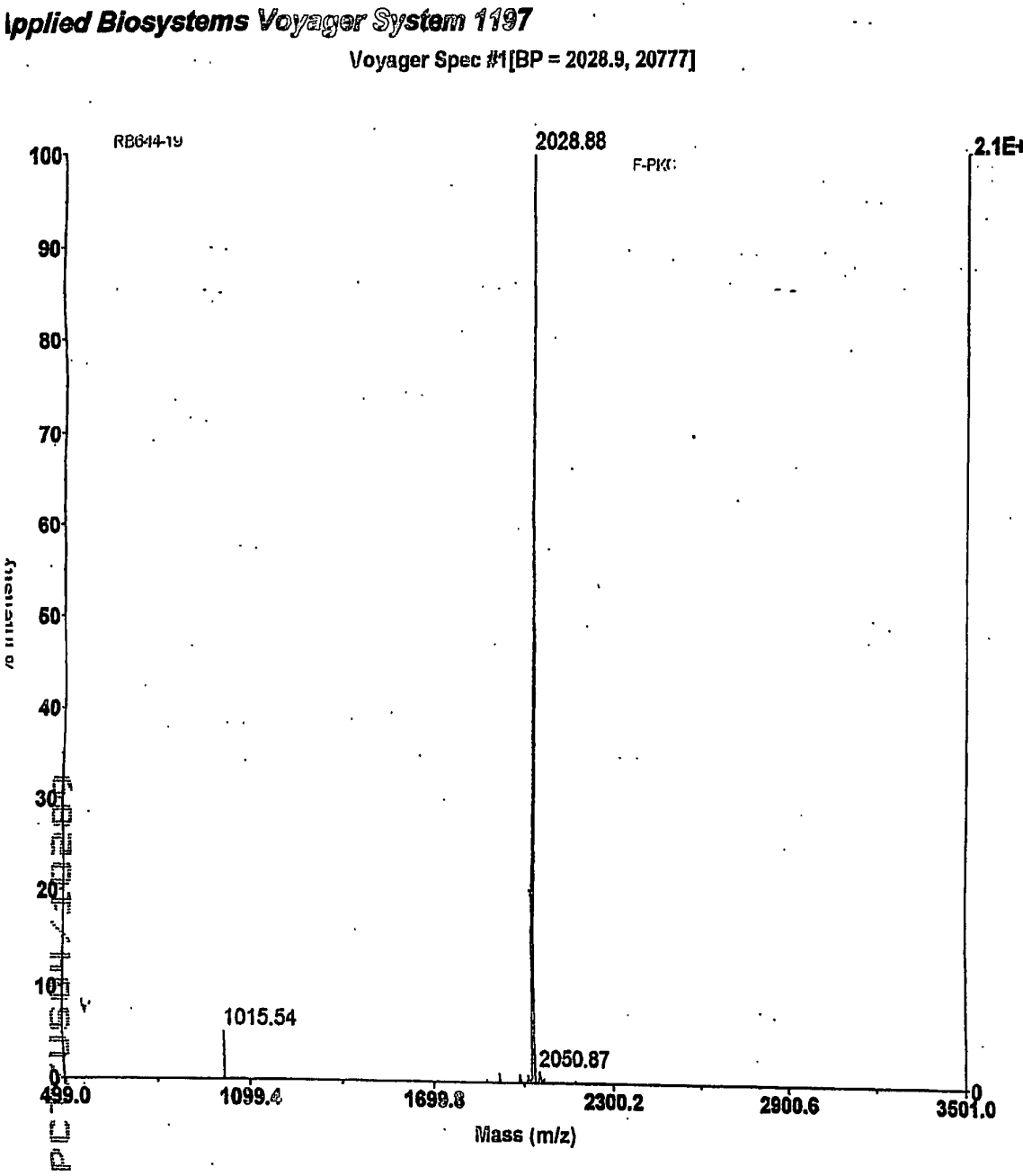
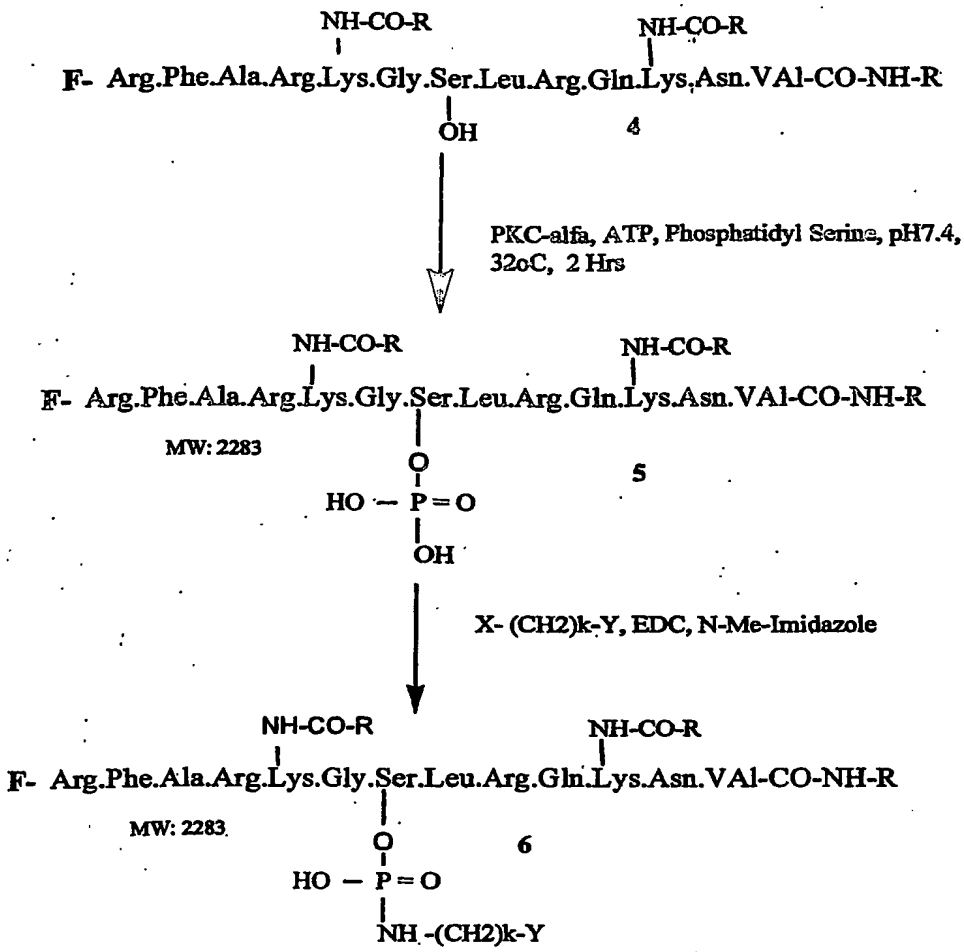


Figure 6. Maldi-Mass spectrum of the PKC –peptide target labeled with fluorescein at its N-terminal for the kinase activities of the isozymes of Protein Kinase C (PKC)



Where k = 1-15
and Y = biotin, biotin-avidin complex, biotin-streptavidin complex, avidin-alkaline phosphatase (AP) conjugate, or unconjugated AP, b-Lactamaze, Casein, or any other large molecular weight, including but not limited to antibodies, and derivatized particles.

Figure 7. Protocol and chemistry of the present invention for the formation of phosphor-amidates used in the detection of phosphoryl groups using the Nucleation Centers and rapid assay methods and phosphoramidate Chemistry I of the present invention

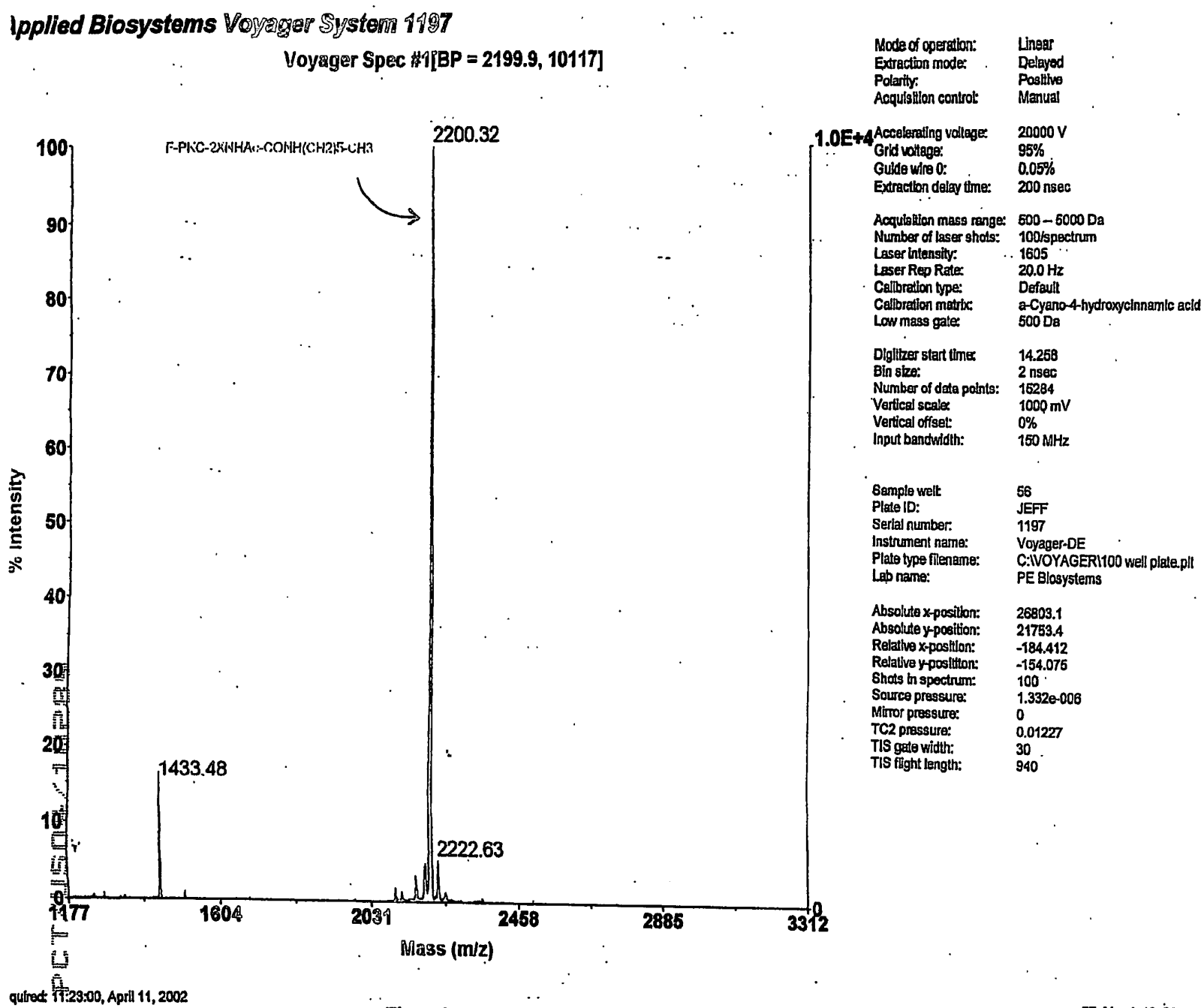


Figure 8a: Maldi-MS of fully protected fluoresceinated PKC peptide target that potential reactive sites blocked as described in figure 5

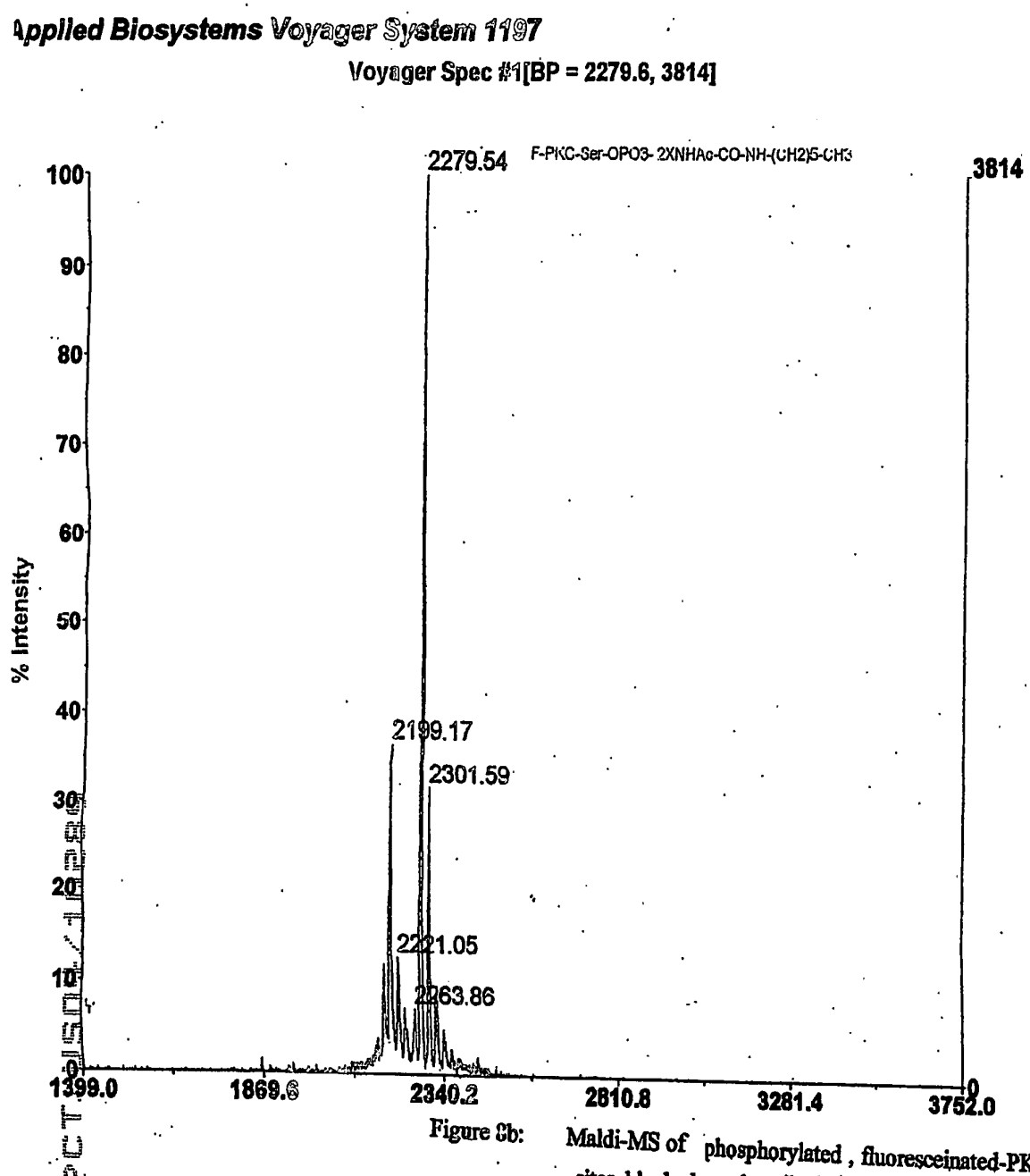


Figure 6b: Maldi-MS of phosphorylated , fluoresceinated-PKC peptide target that had potential reactive sites blocked as described in Example 1 ***before*** the addition of multiplexed Nucleation Centers that had been preformed from avidin and the heterobifunctional biotin linkers of chemistry I.

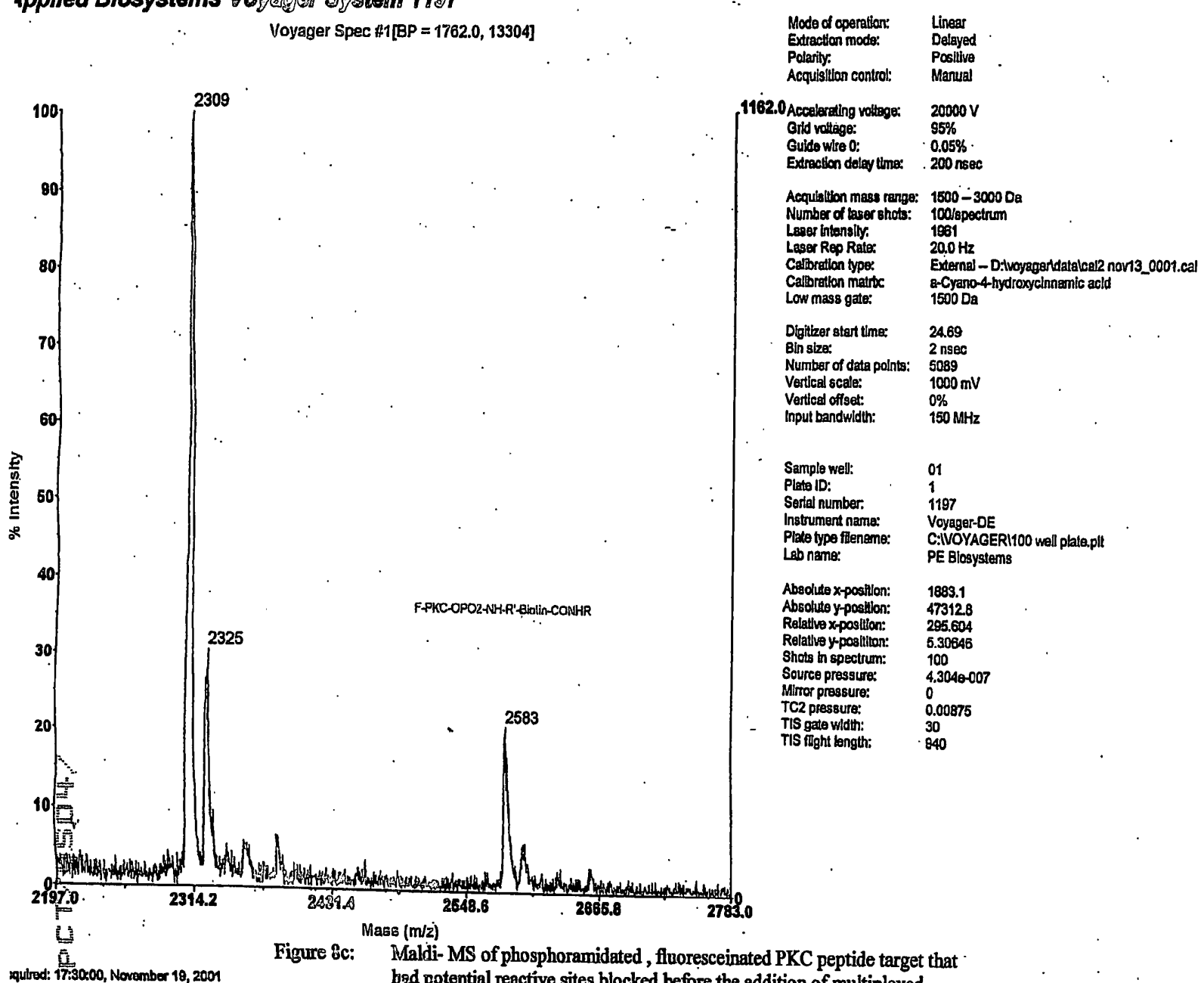


Figure 8c: Maldi- MS of phosphoramidated , fluoresceinated PKC peptide target that had potential reactive sites blocked before the addition of multiplexed preformed Nucleation Centers from avidin and heterobifunctional biotin

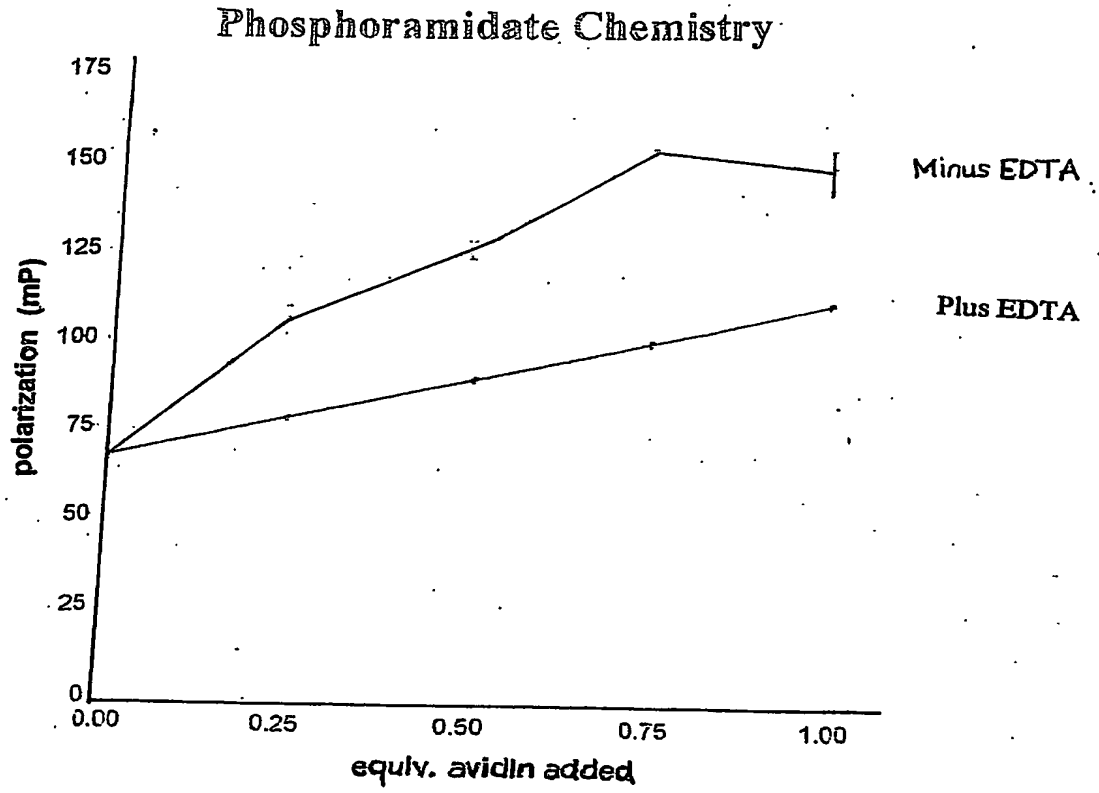
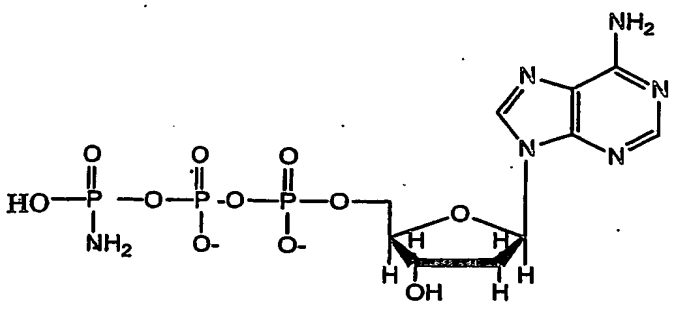


Figure 9. Fluorescence Polarization analysis of the stoichiometry of Nucleation Center Binding. The phosphoramidated PKC peptide target shown in Figure 7 after the addition of varying amounts of multiplexed Nucleation Centers using the linkers of Chemistry I. The two samples differed in that the negative controls were performed in the presence of 5mMolar EDTA which destroys the activity of the kinase.

Structure of γ-NH_2-ATP:



7

Figure 10. Chemical structure of the ATP structural analog , γ-Amino ATP (γ-NH_2-ATP)

Synthesis of γ-Amino-ATP

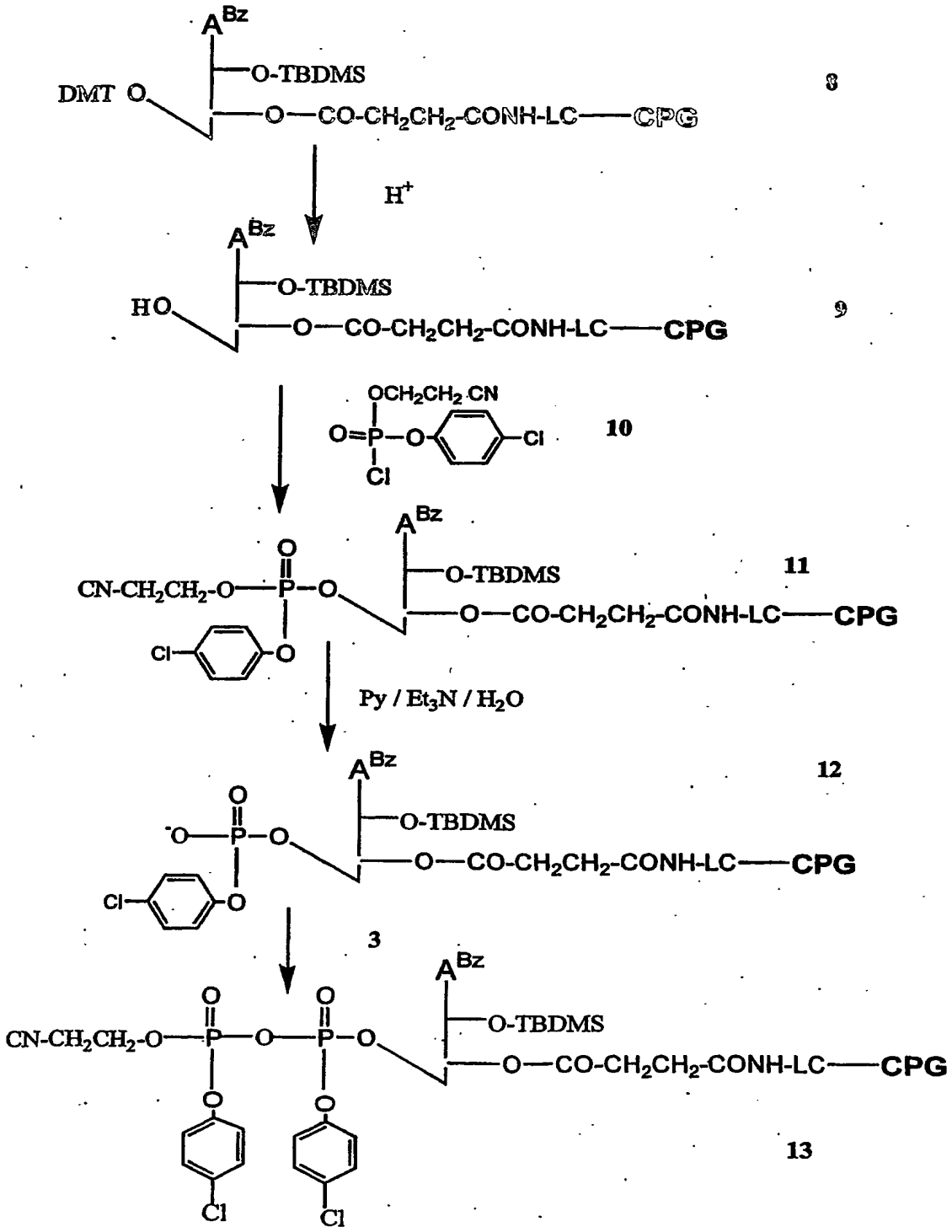


Figure 11. Protocol and chemistry of the present invention for the synthesis of γ-NH2-ATP

Scheme for the synthesis of gamma-Amino-ATP continues----------

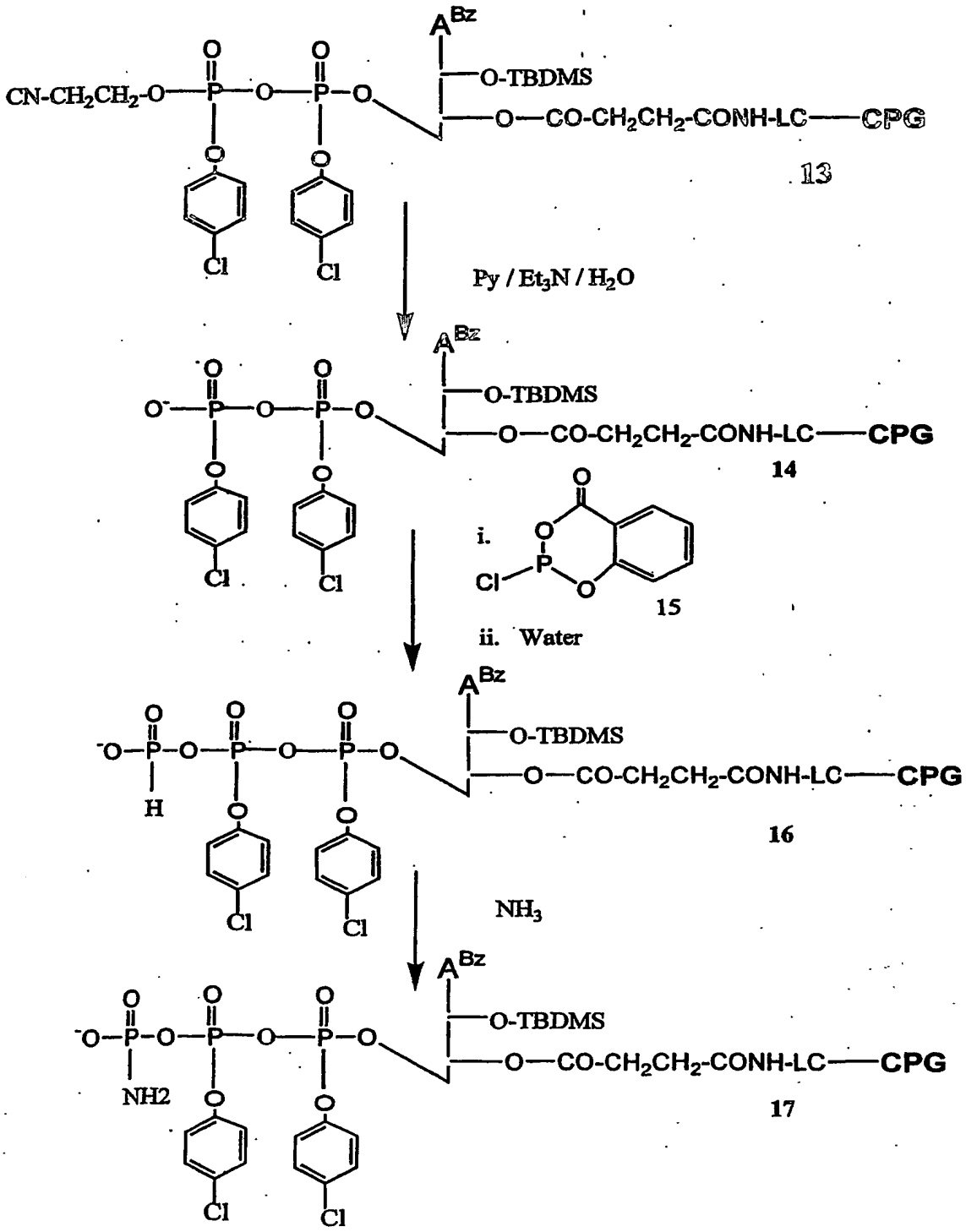
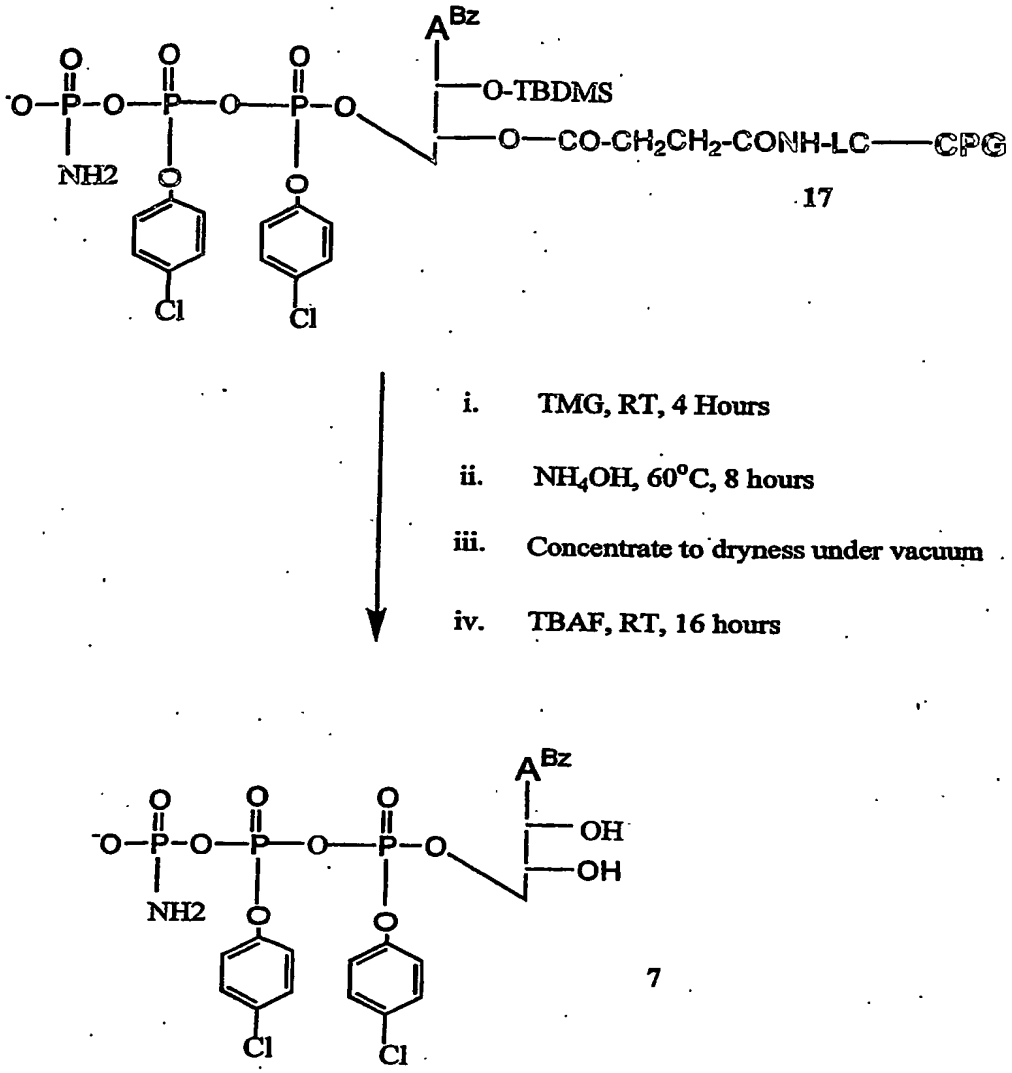


Figure 11: continuation (page 2)

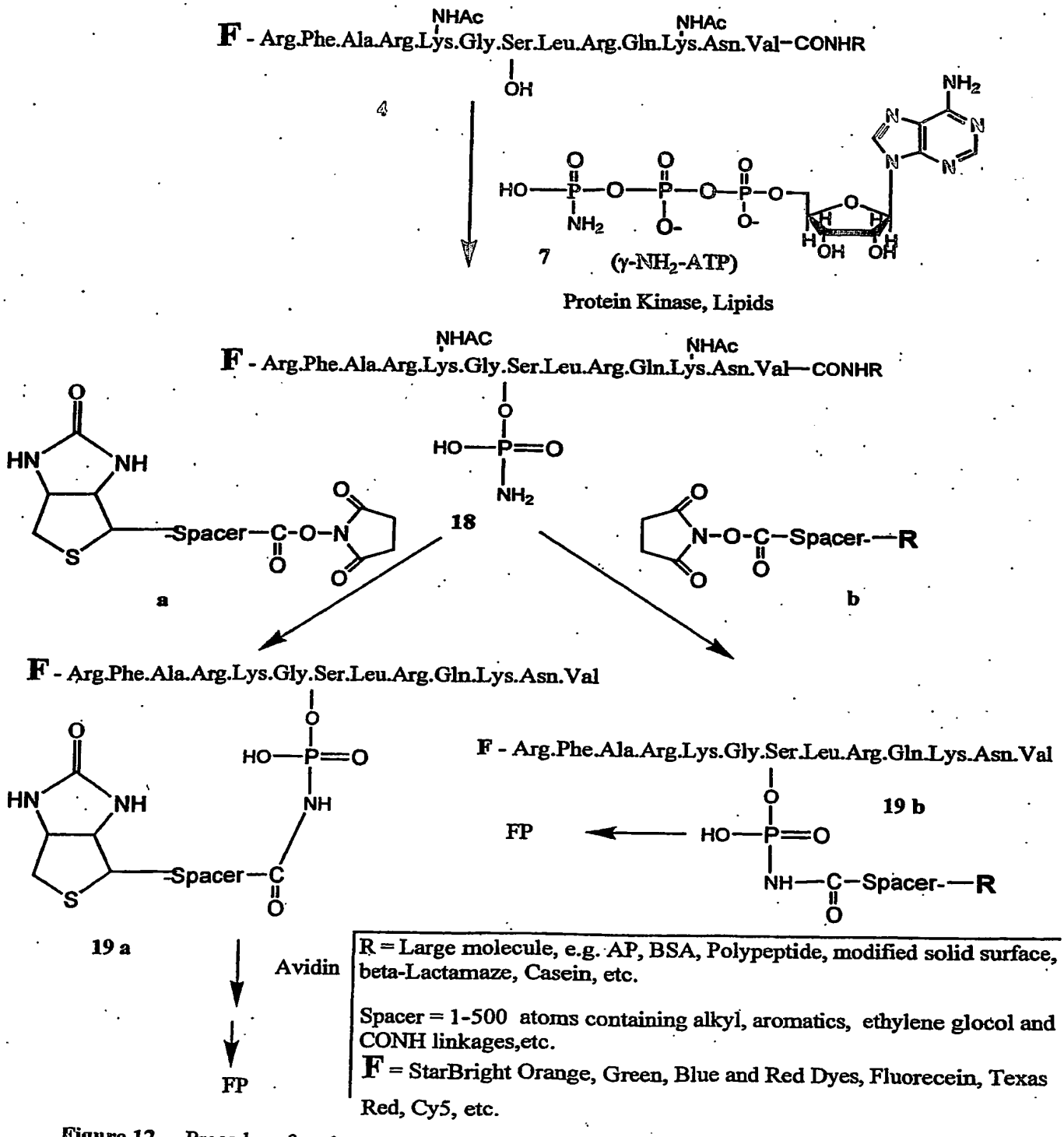
Page 3 of Synthesis of γ-Amino-NH_2



γ-Amino-NH_2

Figure 11: continuation (page 3)

Alternative Approach For Monitoring the Activity of Protein Kinases



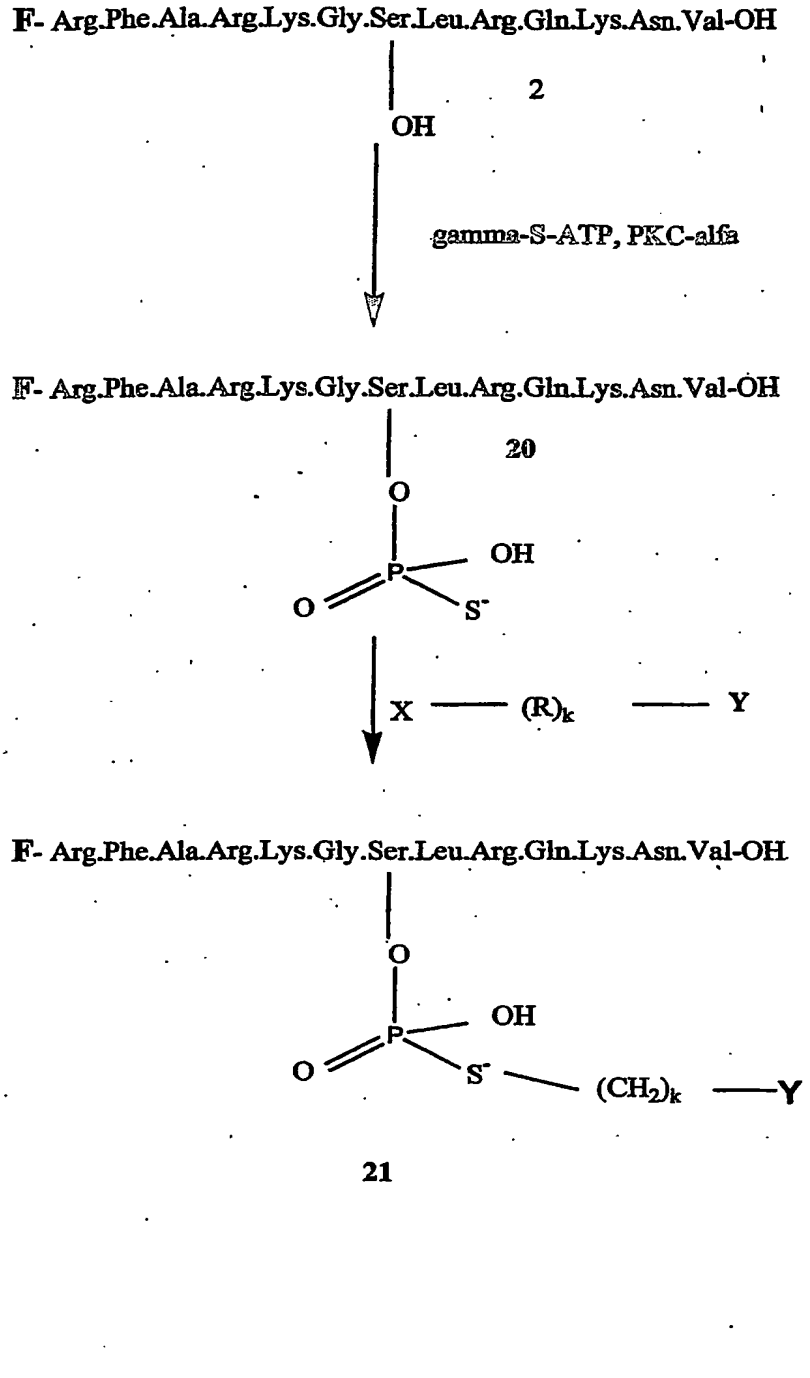
R = Large molecule, e.g. AP, BSA, Polypeptide, modified solid surface, beta-Lactamaze, Casein, etc.

Spacer = 1-500 atoms containing alkyl, aromatics, ethylene glocol and CONH linkages,etc.

F = StarBright Orange, Green, Blue and Red Dyes, Fluorecein, Texas Red, Cy5, etc.

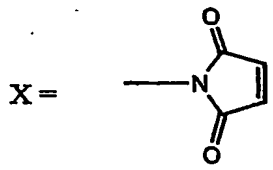
Figure 12. Procedure for phosphoroamidation of fluoresceinated –PKC peptide target using PKC-alpha and γ-NH_2-ATP

Phosphorothioate Chemistry



where k= 1-100
R= Alkyl, alkoxyl, cycloalkanyl, aromatic, heterocyclic, ethylene glycolic, peptidyl, etc

Y= Biotin, Biotin-Avidin, Biotin-Streptavidin, or Large Polymer such as Alkaline Phosphatase (AP), Streptavidin (SA), Casein, glycoprotein, IgG, enzyme, DNA, RNA with or without conjugation to Avidin



X = I-CH_2CONH--
= Br-CH_2CONH-
= S

Figure 13. Protocol and chemistry of the present invention for phosphorothiolation and detection of fluoresceinated PKC-peptide target using the single step, nucleation effect rapid assay method and Chemistry III of the present invention

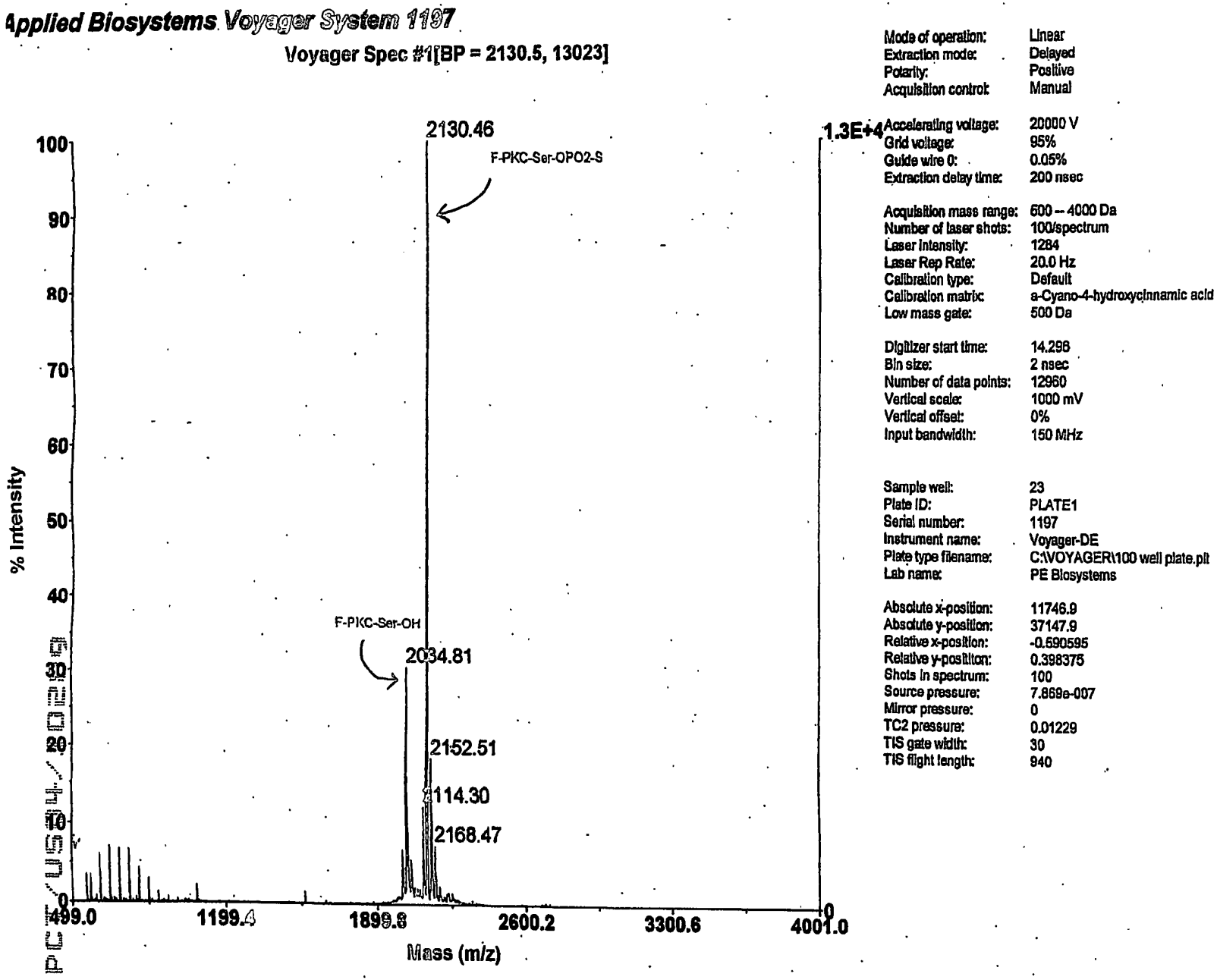


Figure 14 (a): Maldi-MS of Phosphorothiolated fluoresceinated -PKC peptide target

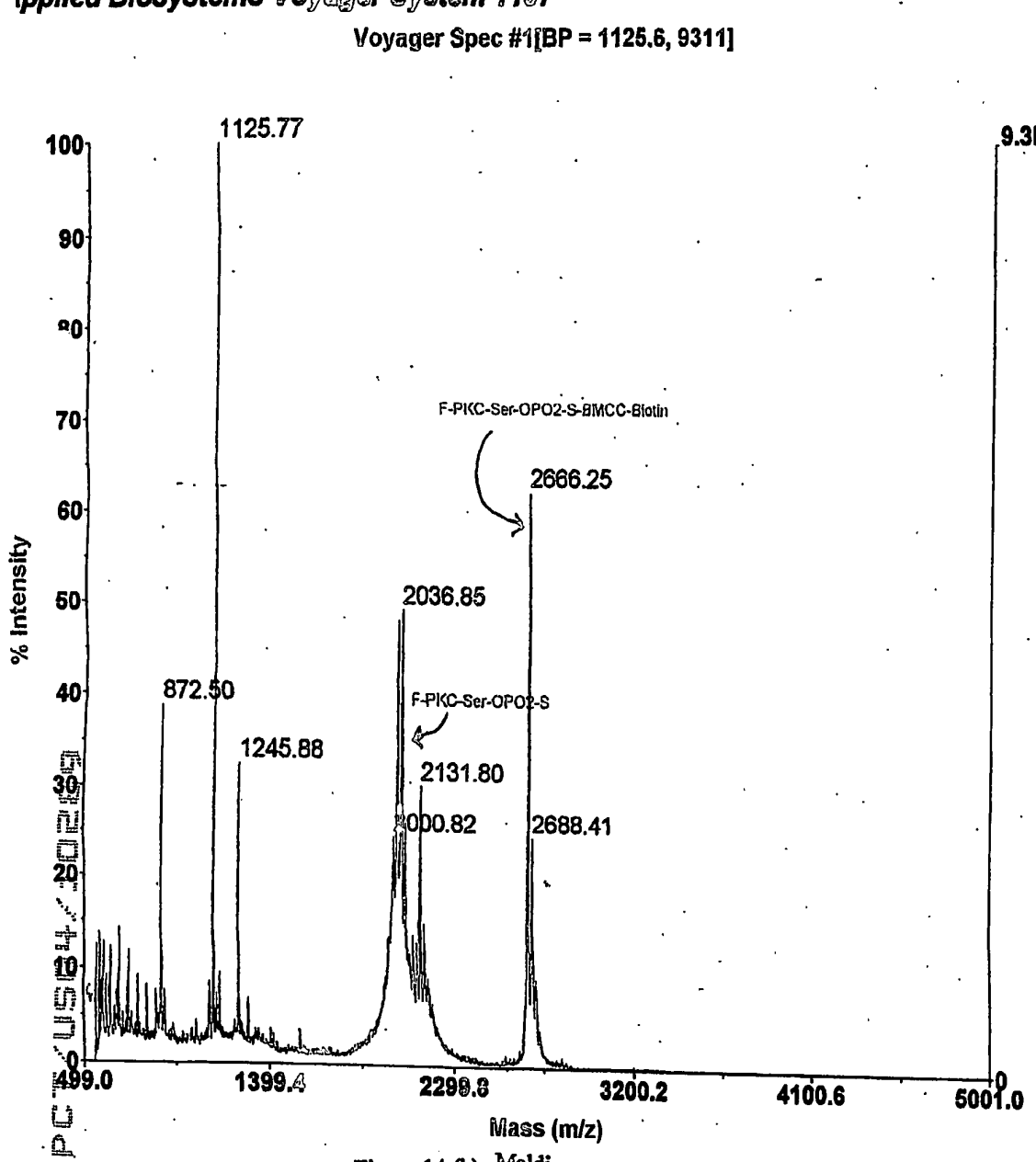
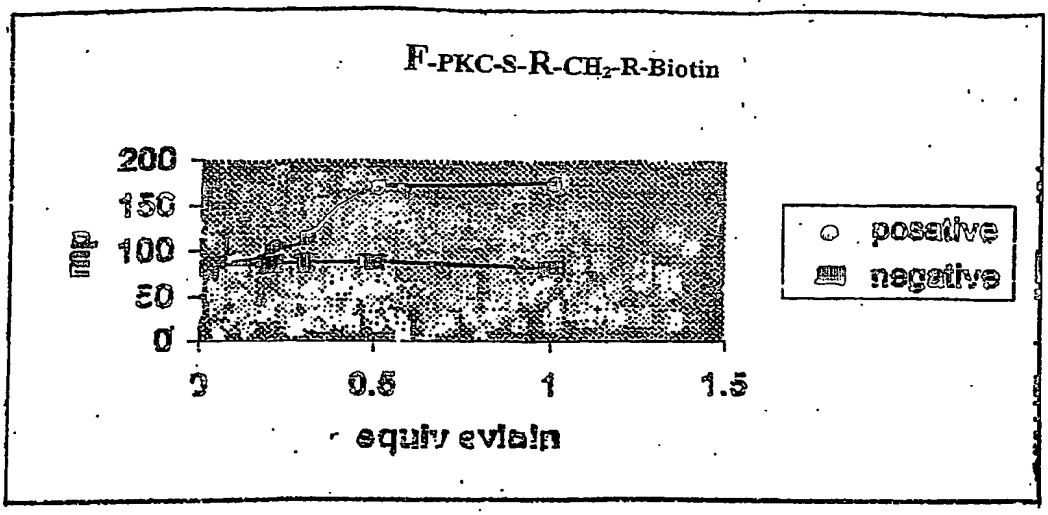
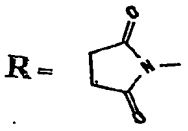


Figure 14 (b): Maldi mass spectrum of the same phosphorothiolated, fluoresceinated PKC peptide target 15 minutes after addition of equimolar equivalents multiplexed Nucleation Centers prefomed from avidin and the hetero-bifunctional linkers





R = Long Chain alkyl

Figure 14 (C) Fluorescence polarization analysis of the same sample used to generate the spectrum of 14(b), above, showing the titration with multiplexed Nucleation Centers that wer prefomed from avidin and the hetero-bifunctional linkers, maleimido BMCC-bioti

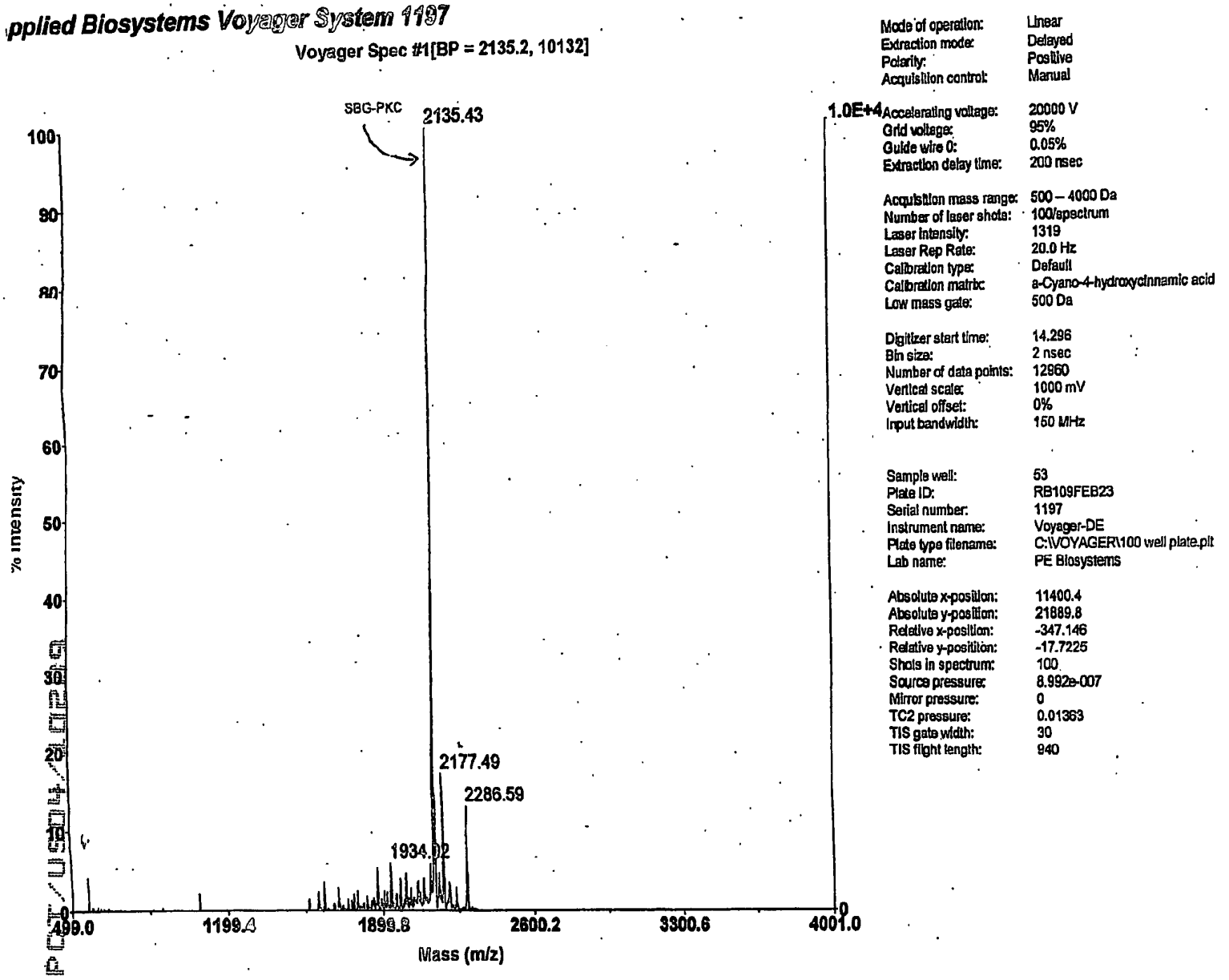


Figure 15 (a): Maldi-MS of StarBright Green -PKC peptide target before the addition of enzyme and donor

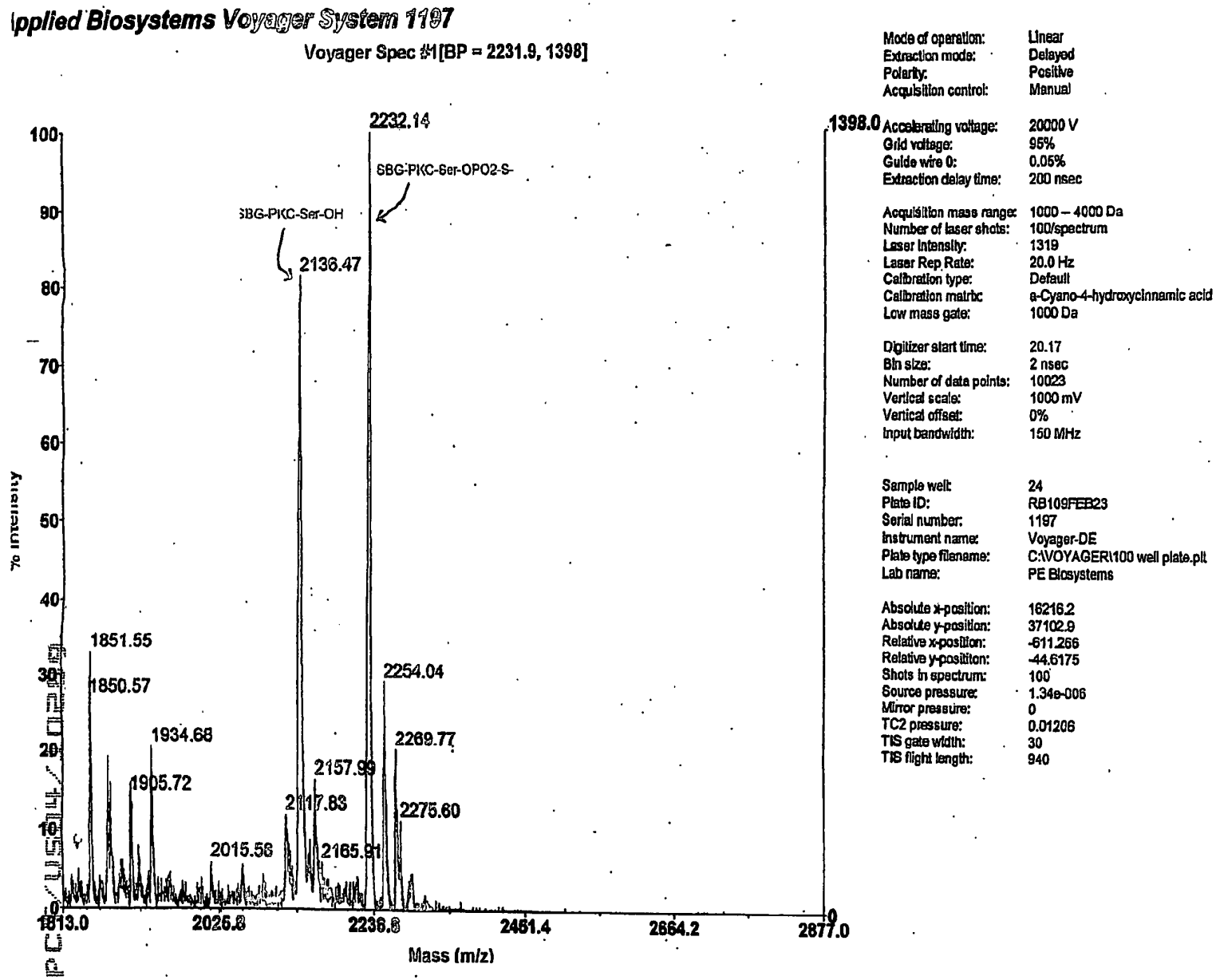


Figure 15 (b): Maldi-MS of Phosphorothiolated StarBright Green -PKC peptide target with enzyme and γ-S-ATP

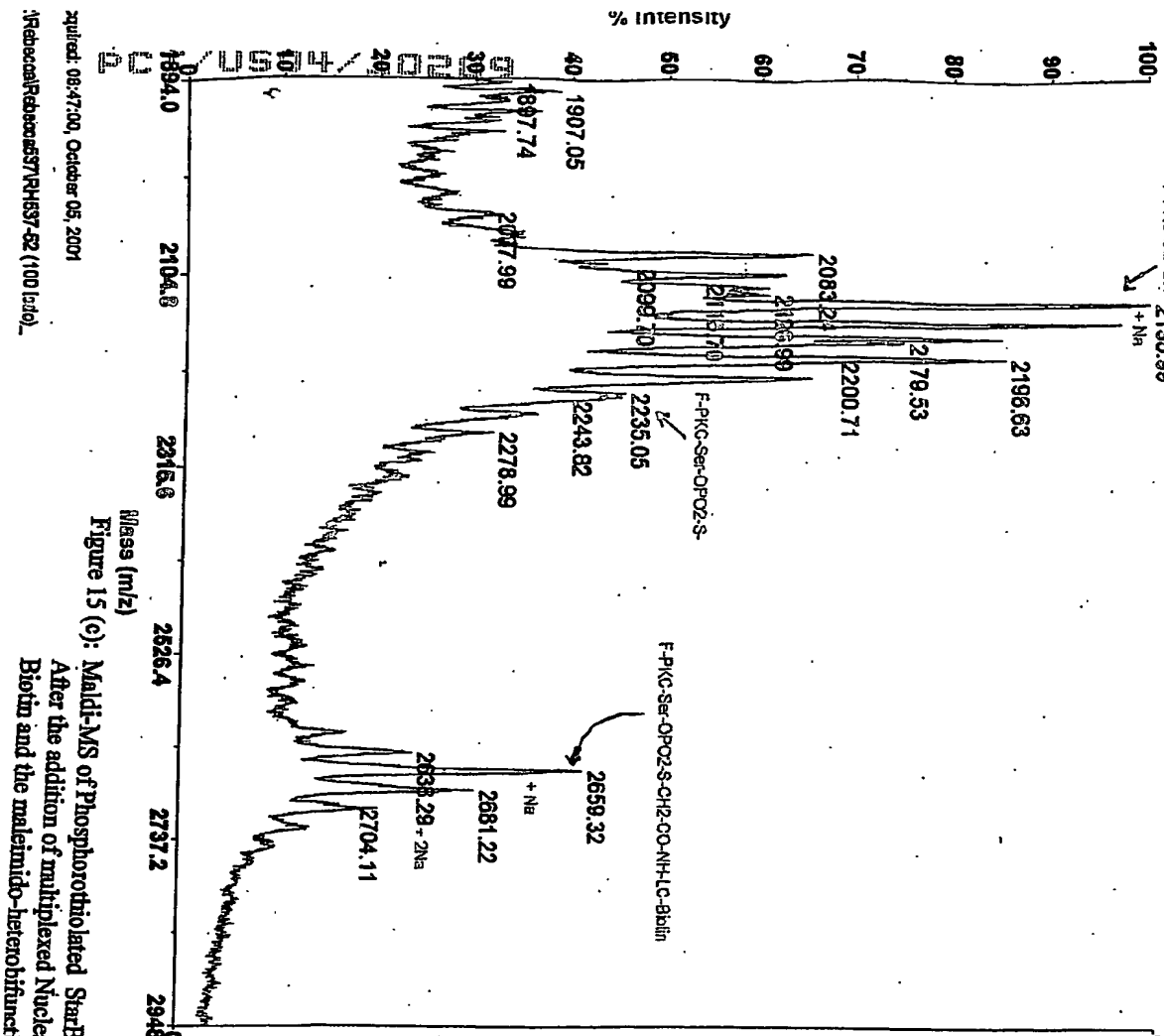


Figure 15 (c): Maldi-MS of Phosphorothiolated StarBright Green -PKC peptide target After the addition of multiplexed Nucleation Centers preformed from Biotin and the maleimido-heterobifunctional linkers

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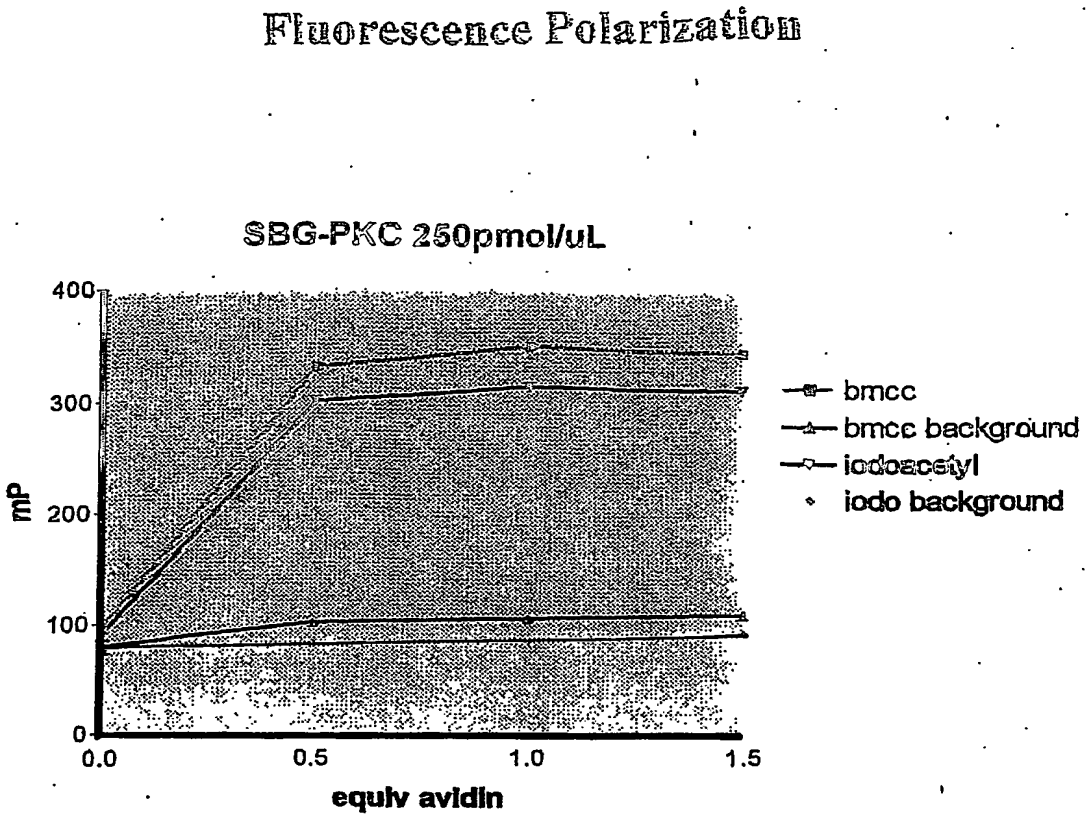

Figure 16. Fluorescence Polarization Analysis of the extent of the reaction of multiplexed Nucleation Centers prefomed from avidin and multiple heterobifunctional linkers bearing biotin at one terminus and maleimido- (b[illegible]) and iodoacetamido- reactive groups at the other (purple line). The StarBright Green –PKC peptide target was phosphorylated by PKC-theta using γ-S-ATP as the donor.

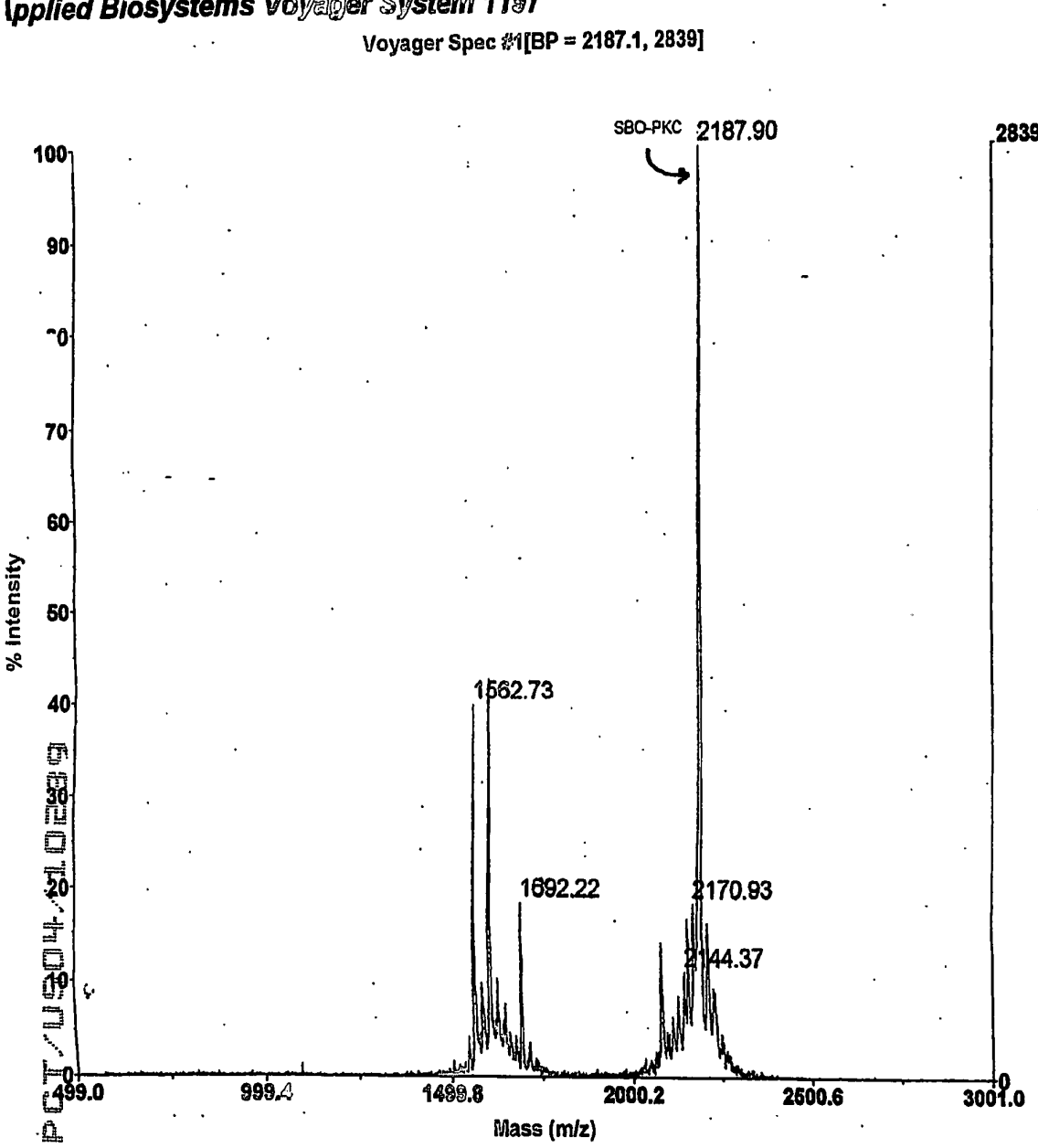

Figure 17 (a): Maldi-MS of StarBright Orange -PKC peptide target before the addition of enzyme and donor

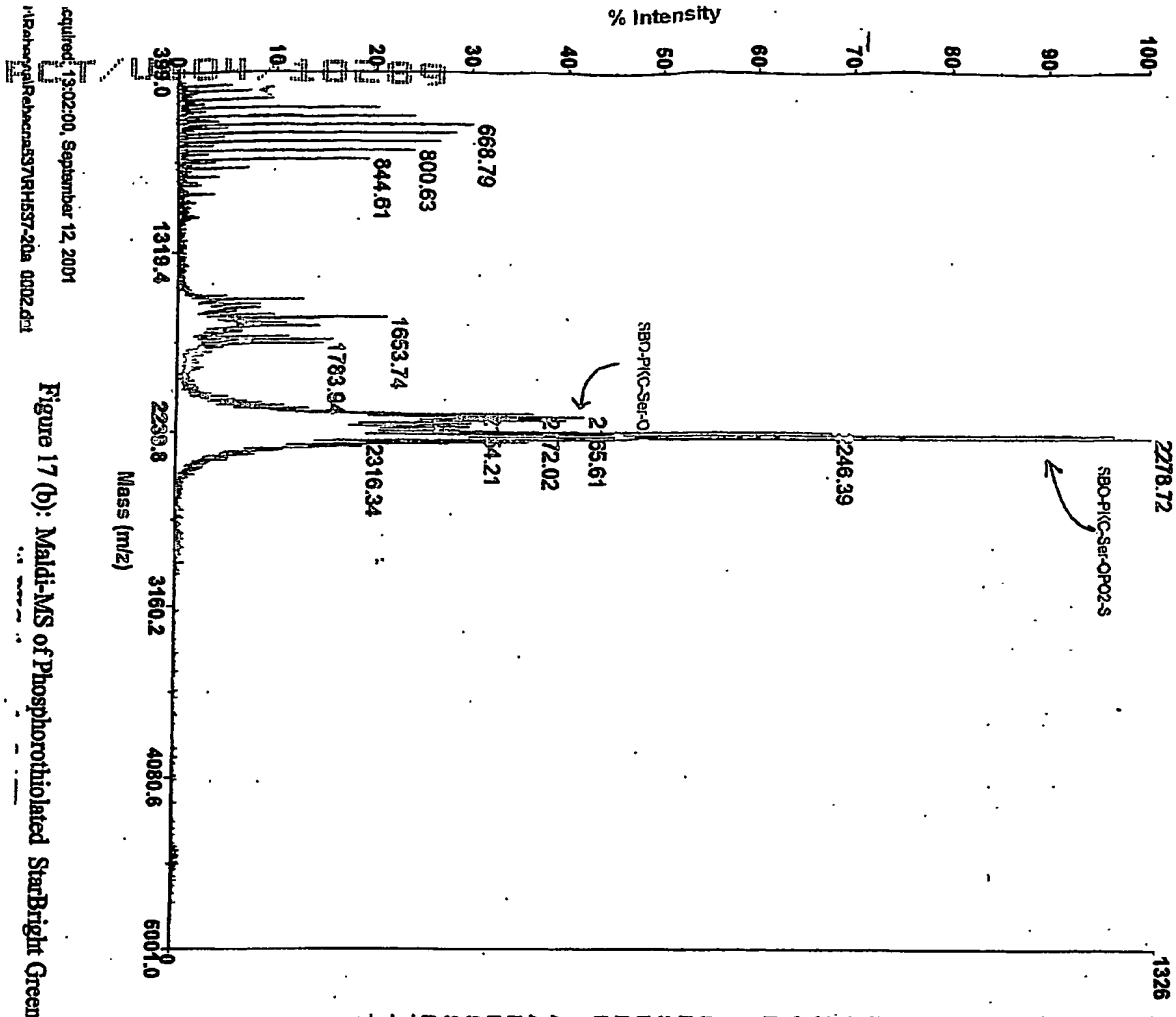

Figure 17 (b): Maldi-MS of Phosphorothiolated StarBright Green -PKC peptide target

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'igure 18 (a) : **Fluorescence Polarization of SBO-PKC-Ser-OPO2-S-BMCC-LC-Biotin after the addition of Avidin**

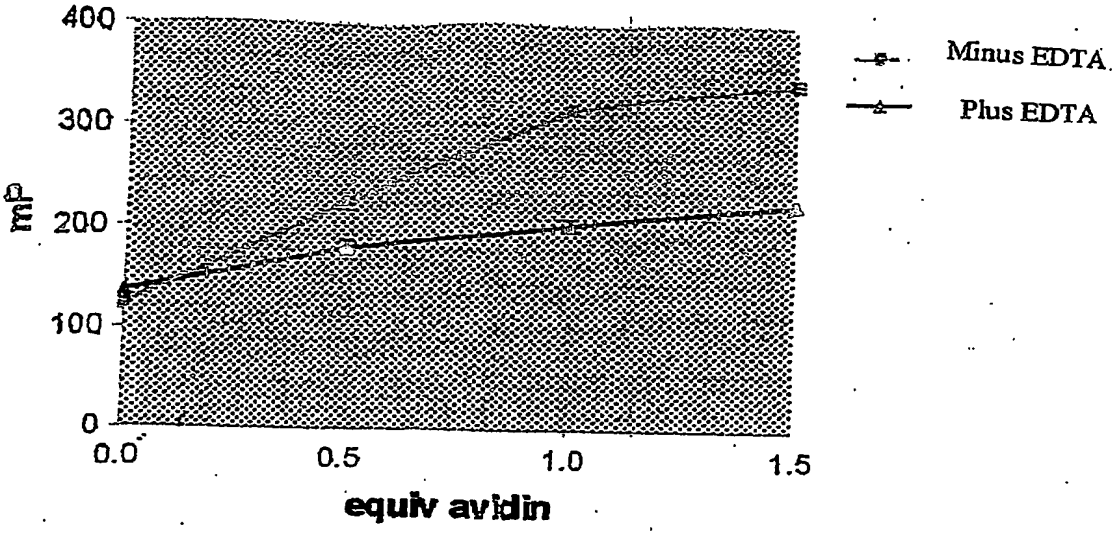
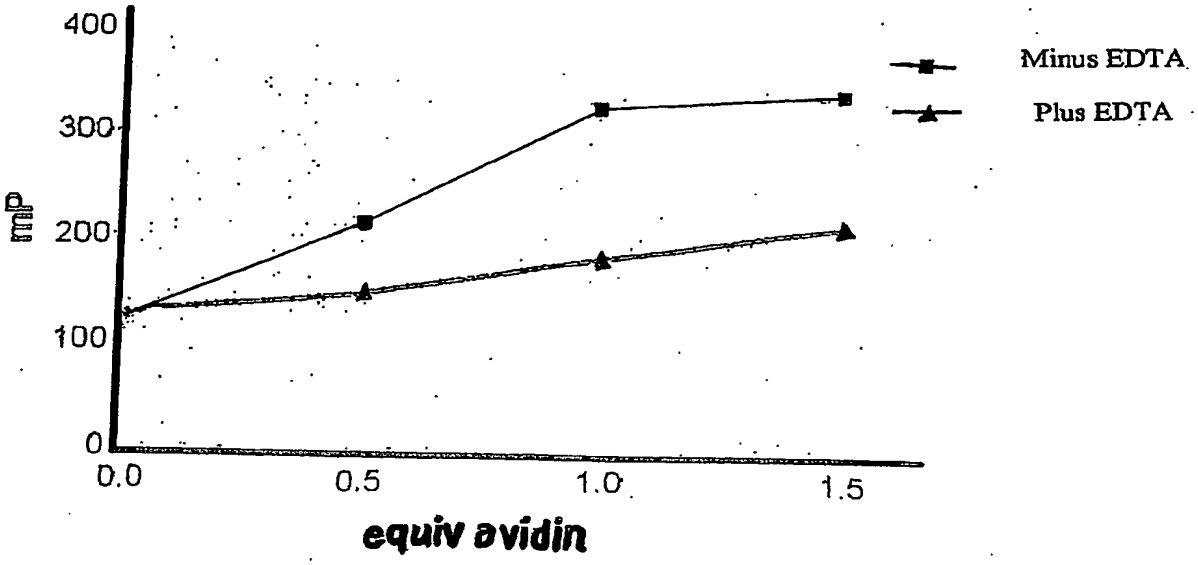
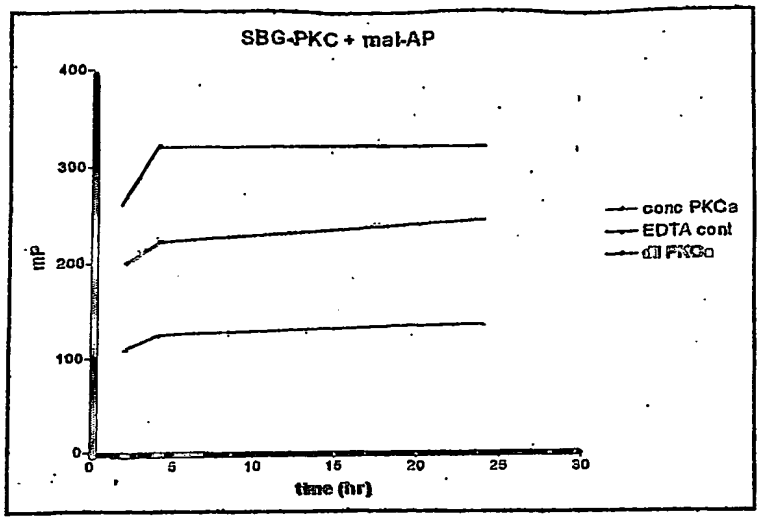


Figure 18 (b) : **Fluorescence Polarization of SBO-PKC-Ser-OPO2-S-Iodoacetyl-LC-Biotin after the addition of Avidine**



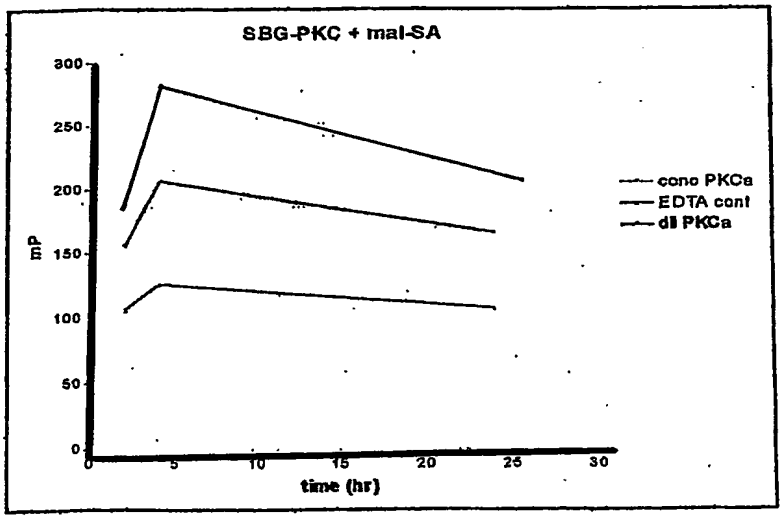
Fluorescence Polarization Using Large Molecules

(a)



time (hr)	conc PKCa	EDTA cont	dil PKCa
2	267	110	200
4	321	125	223
24	321	136	245

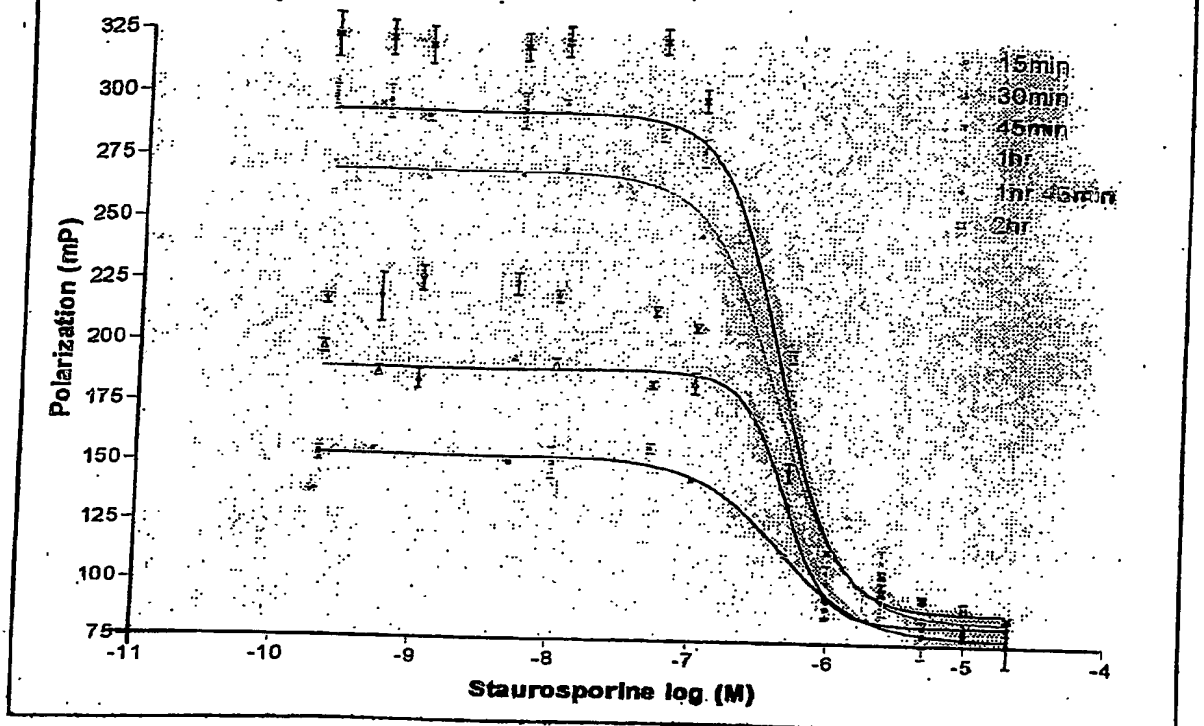
(b)



time (hr)	conc PKCa	EDTA cont	dil PKCa
2	187	106	156
4	281	126	207
24	213	107	166

Figure 19: Fluorescence polarization of analysis of phosphorothiolated SBG-PKC after the addition of multiplexed Nucleation centers comprised of Alkaline Phosphatase, figure (a), and Streptavidine, figure (b), bearing multiple maleimido groups capable of reacting with the phosphorothiolated peptide described in figure 13.

Figure 20: Fluorescence polarization analysis of the inhibition of phosphorothiolation of SBG-PKC target by Protein Kinase C-α by Staurosprine (1% DMSO) measured using multiplexed Biotin Nucleation Centers bearing maleimido groups



EC50	4.39E-07	4.76E-07	4.40E-07	4.01E-07	4.16E-07	4.39E-07
KI	1.25E-07	1.36E-07	1.26E-07	1.15E-07	1.19E-07	1.26E-07

Fluorescence Polarization (mp)

15min		30min		45min		1hr		1hr45		2hr	
67	76	66	85	65	82	71	85	80	83	87	86
80	78	83	81	80	77	92	81	91	77	93	89
87	69	85	74	83	81	85	83	93	92	93	96
87	99	96	101	95	98	107	112	105	115	103	106
93	85	94	86	93	91	99	101	108	101	114	113
113	111	134	133	143	151	167	171	174	182	193	198
142	143	168	179	208	205	243	245	283	272	309	294
154	158	181	184	211	215	267	273	282	287	327	317
143	156	189	193	216	221	277	263	298	293	327	315
150	149	193	193	226	219	270	265	300	286	313	324
151	152	180	188	230	220	262	269	291	288	326	311
155	152	185	189	207	227	268	270	289	302	314	328
155	149	199	194	218	214	276	267	302	292	331	313
149	154	183	191	217	223	271	269	291	294	312	326
74	78	71	83	74	80	82	89	75	84	82	86
152	145	180	191	212	210	263	268	282	286	307	319